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13. ABSTRACT ***(Maximum 200 Words)***

Maspin is a unique member of the serpin family that shares extensive homology with monocyte-neutrophil elastase inhibitor, PAI-2 and other serpins. Initially identified as a class II tumor suppressor gene, maspin has been shown to inhibit invasion and motility of mammary tumors. When maspin gene was introduced into breast tumor cells, it was demonstrated that tumor transfectants expressing maspin exhibited significant decrease in breast tumor growth and metastasis in nude mice. Maspin gene expression is not detected in most breast tumors and loss of its expression is correlated with tumor invasiveness. Maspin is also found to be a potent angiogenesis inhibitor. Based on the previous work by my laboratory and other colleagues, I hypothesize that maspin possesses multiple functionality that requires its interaction with multiple target proteins. Maspin could act intracellularly or be secreted to act in a paracrine fashion on adjacent cells. I propose to isolate maspin target(s) by combined genetic and biochemical approaches. Once the target is identified, deletion and mutagenesis studies will be carried out to identify the functional domain of maspin that is responsible for such protein-protein interaction. Finally, such interaction will be verified in mammalian cells.

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Ming Zhang, Ph.D. 8/23/03

PI - Signature Date

Table of Contents

Introduction

Background: Maspin is a unique member of the serpin family that shares extensive homology with monocyte-neutrophil elastase inhibitor, PAI-2 and other serpins. Initially identified as a class II tumor suppressor gene, maspin has been shown to inhibit invasion and motility of mammary tumors. When maspin gene was introduced into breast tumor cells, it was demonstrated that tumor transfectants expressing maspin exhibited significant decrease in breast tumor growth and metastasis in nude mice. Maspin gene expression is not detected in most breast tumors and loss of its expression is correlated with tumor invasiveness. Maspin is also found to be a potent angiogenesis inhibitor. Other than its anti-tumor function, maspin clearly plays an important role in normal development. We have demonstrated that transgenic mice overexpressing maspin in mammary gland inhibit alveolar development and mammary cell differentiation, and maspin transgenic mice had a protective role against breast tumor growth and metastasis. Overall, maspin is a gene with an important function in both normal mammary development and breast tumorigenesis.

Objective/Hypothesis: Based on the previous work by my laboratory and other colleagues, I hypothesize that maspin possesses multiple functionality that requires its interaction with multiple target proteins. Maspin could act intracellularly or be secreted to act in a paracrine fashion on adjacent cells. I propose to isolate maspin target(s) by combined genetic and biochemical approaches. Once the target is identified, deletion and mutagenesis studies will be carried out to identify the functional domain of maspin that is responsible for such protein-protein interaction. Finally, such interaction will be verified in mammalian cells.

The specific aims for this three-year proposal are:
(1) Identification of maspin target protein by genetic and biochemical approaches, and (2) characterization of the functional domain of maspin responsible for protein-protein interaction.

Body

Materials and methods

Yeast library

A Hela cDNA library has been obtained from Dr. Roger Brent at Harvard Medical School. Yeast EGY48 cells were avalaible at the PI's laboratory.

Yeast two-hyb assay

Yeast EGY48 cells will be transformed with LexA/maspin fusion plasmid and a LexA/lacZ reporter and selected on yeast Ura- and His- plates. The transformed yeast cells will be used to screen a Hela library consisting of B42/Hela cDNA fusions. The putative positive clones should be able to grow in the Ura-, His-, Trp- and Leu- plate containing galactose as the carbon resource and become blue in the presence of X-gal.

Antibodies
Antibody for HA tag of the fusion protein was purchased from Sigma. Polyclonal anti-maspin antibody was made by Zymed, Inc. as a custom service. All secondary antibodies were purchased from Zymed, Inc.

Western analysis
Total yeast proteins were isolated from cells. Protein extracts were prepared by lysing the cells in RIPA buffer. Total 100 ug protein extract will be loaded for electrophoresis. Western blot analysis will be carried out using HA and maspin antibodies to dectect maspin and its interacting proteins.

Radioactive receptor binding assay
The GST-maspin protein will be made in E.coli and purified by glutathionine affinity column. Protein will be labeled by 125-iodine. MDA231 breast tumor cells will be used for binding assay. Radiolabeled maspin will be incubated with live cells for a time course between 45 mins to 90 mins at 4 0C to prevent degradation and endocytosis of maspin ligand. After washing, the amount of labeled protein on cell surface will be counted in gama counter to determine the binding kinetics.

Results and Discussion

Task 1. Identification of putative receptor of maspin
Months 1-12.

Identifying putative receptor of maspin

We have carried out some receptor binding experiment. Maspin proteins made in E.coli were labeled by 125-I and used in the cell based binding assay, using MDA231 breast cancer cells. The condition is described in the materials and methods. Briefly, radiolabeled maspin was incubated with live cells for a time course between 45 mins to 90 mins at 4 0C to prevent degradation and endocytosis of maspin ligand. The reaction was briefly washed with PBS/0.1% BSA solution. Different cold/hot maspin concentrations (cold maspin for competition purpose) were used to determine the binding kinetics. Preliminary data show that although there was increased binding of maspin to cells at higher dose of labeled protein, the non-specific binding also increases (see fig.1). This indicates that there is high stickyness of maspin protein to cell matrix. Currently, we are trying to remove the non-specific binding and determine Kd and Bmax by Scatchard analysis. If the maspin binding constant is too small, the cell binding assay may not be appropriate since washing and cell endocytosis may disrupt maspin/receptor binding, resulting in non-specific binding or data not reliable for interpretation. In this case, we plan to isolate plasm membrane fraction from MDA231 cells and use the membrane directly for ^{125}I binding assay.

Task 2. Identification of maspin target by yeast two-hybrid system
Months 1-24.

We have started to use the Hela cDNA library for yeast two hybrid screening. Briefly, yeast EGY48 cells were transformed with LexA/maspin fusion plasmid and a LexA/lacZ reporter and selected on yeast Ura- and His- plates. The transformed yeast cells were then transformed with Hela library consisting of B42/Hela cDNA fusions. In the last eight months, we have obtained over 200 candidate clones that were able to grow in the Ura-, His-, Trp- and Leu- plate containing galactose as the carbon resource and became blue in the presence of X-gal. Plasmids were isolated from these clones and retransformed to yeast cells to confirm the ability of these plasmid to confer x-gal inducibility. Over the 200 primary clones, 30 or so secondary clones were identified. Many plasmid sequenced contained nonsense coding sequences and were further eliminated. Currently, we are testing some of the isolated clones to verify whether they are true maspin interacting partner. In vitro IP-western analysis will be done to further confirm the interaction. The identity of a dozen of selected clones are listed below (Fig.2).

Task 3. Identification of maspin target by biochemical approach
Month 6-24

We have recently started the task 3 to identify the maspin target by a biochemical approach. In this case, we used a GST-maspin protein to incubate with the extract of MDA-231 and MCF10A cells. MDA-231 mammary tumor cells were chosen over MDA-435 tumor cells because of the concern that the later may not be a good mammary tumor cell strain. MCF10A is a normal immortalized human mammary epithelial cell line which expresses maspin by itself. Preliminary experiment data using MCF10A cell extract indicate two distinctive protein bands were present in GST-maspin pull down assay but were absent in the control GST-treated sample. Figure 3 shows the SDS gel stained by silver staining method. Arrows indicated the potential target proteins. The identity of these proteins will be revealed by peptide sequencing once we obtain enough materials.

Key research accomplishments

Key personnel have been recruited to initiate the study as proposed in the grant. We have initiated the receptor binding assay and the yeast two hybrid screening has identified several candidate clones (Fig.2). In addition, in vitro biochemical analysis has identified two protein bands that may be the targets of maspin protein (Fig.3).

Reportable outcome

None

Conclusion

None

Reference

None

Appendices

Figures 1-3

Fig.1. Binding of I^{125}-his-maspin to MDA-MB-231 cells surface

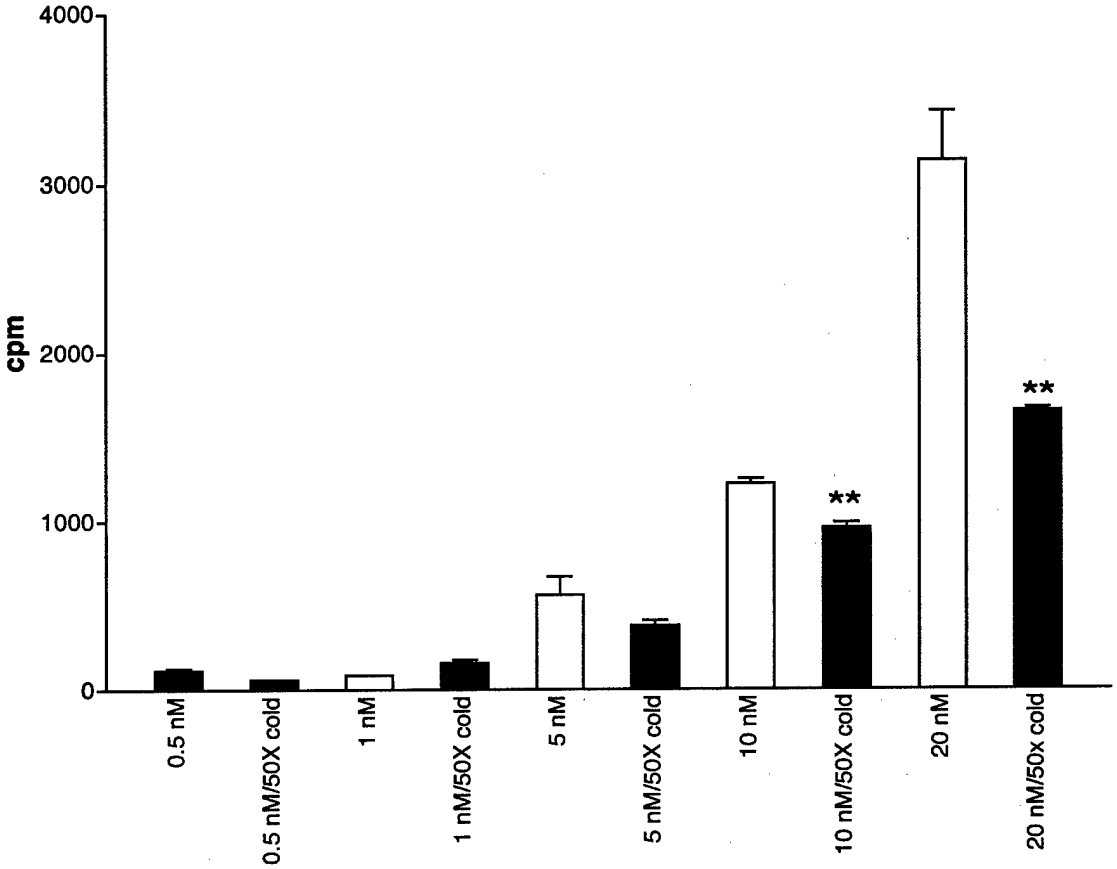

Fig.2. Putative maspin target proteins identified from yeast two-hybrid assay

1.yZL1-2	chaperonin containing TCP1 subunit 7
2.yZL1-10	ferritin
3.yZL1-14	triosephosphate isomerase 1
4.yZL1-57	elongation translation factor 1
5.yZL2-3	phosphoglycerate (PGK1)
6.yZL2-44	CRM1 protein
7.yZL3-27	nucleolar protein ANKT
8.yZL1-3	hypotheltical protein
9.yZL1-47	histon H2A F/Z variant (H2AV)
10.yZL3-30	SUMO-1
11.yZL1-5	muscle specific gene M9
12.yZL2-39	peroxiredoxin

Fig.3 Maspin immunoprecipitation in MCF10A cells silver staining